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TITLE: Molecular Determinants Fundamental to Axon Regeneration after SCI

PRINCIPAL INVESTIGATOR: Jeffrey Alan Plunkett, Ph.D.

CONTRACTING ORGANIZATION: St. Thomas University, INC.
Opa Locks, FL 33054

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Opa Locka, FL 33054

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

There are no therapies available that restore motor impairments resulting from spinal cord injury (SCI). Soldiers with SCI are permanently paralyzed and in need of lifelong care. Promoting axon regeneration after SCI may lead to the formation of axon circuits that may be involved in (or recruited for) motor functions. In the mammalian spinal cord, axon regeneration is frustrated by inhibitors such as chondroitin sulfate proteoglycans (CSPGs) expressed by reactive astrocytes present at the injury site. In adult zebrafish, Danio rerio, some brainstem neurons are able to grow their axon beyond a spinal cord injury, even though inhibitory CSPGs are present. Based on these findings we have developed an overall working hypothesis that the ability to grow an axon over CSPGs is intrinsic to the zebrafish brainstem neurons and entails the expression of a distinct set of genes. In Phase 3, we propose to employ an in vitro model system to determine the relationship between L1.1 and the CSPG neurocan, on axon growth from primary brainstem neurons from adult zebrafish (Specific Aim 1). We also will examine in vivo the role of PTP σ in inhibition of axon regeneration (Specific Aim 2). In addition, we propose to determine the effects of identified transgenic over expressing genes crucial for axon regeneration (Specific Aim 3). The results of the proposed experiments will provide information that may serve as the basis for the development of tailored strategies to promote axon regeneration across injury sites in the spinal cord. The PI is Jeffrey Plunkett, Ph.D.

15. SUBJECT TERMS

None provided.

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Contract # W81XWH-11-1-0645

Title: Molecular Determinants Fundamental to Axon Regeneration after SCI

PI: Dr. Jeffrey Alan Plunkett

Scientific Progress from Sept. 1, 2012 – Aug.31, 2013 (Months 13-24)

Introduction:
This work represents the third phase in our overall strategy to develop effective treatments for the injured spinal cord. The experiments proposed here continue in the investigations in previous phases and build upon the knowledge that was gathered. We will use an *in vitro* and *in vivo* model system to conduct mechanistically-driven experiments that address the overall hypothesis that *the axon growth response in the injured zebrafish spinal cord is intrinsic to brainstem neurons and entails the expression of a distinct set of genes.* In this stage of our approach, we expect that the results from **Specific Aim 1** will demonstrate that increased levels of L1.1 will improve axonal regeneration over neurocan. We further anticipate that the data obtained in **Specific Aim 2** will demonstrate that regeneration of descending brainstem axons across a CSPG-rich transection site will depend on the availability of PTPσ, a CSPG receptor and that the axonal regeneration response is reversely correlated with the availability of PTPσ. The results from the experiments proposed in **Specific Aim 3** are expected to demonstrate that over expression of genes that are involved in axonal extension in the injured spinal cord will enhance axonal regeneration which will correlate with functional restoration. The overall goals of the proposed experiments are to validate our results from the previous studies, to further establish and employ our *in vitro* and *in vivo* model systems, and to further develop our mechanistic-based models for the successful and failed axonal regeneration response observed in the injured adult zebrafish spinal cord.

Body:

SOW Plunkett Lab:
<u>Specific Aim 1:</u> To determine axon growth from cultured adult zebrafish brainstem neurons with increased L1.1 levels over a neurocan substrate.

L1.1 is the zebrafish homolog of L1, a growth-promoting factor in mammals, and known to be involved in axonal regeneration in adult zebrafish[1]. In our previous studies we are studying if down regulation of L1.1 is implicated in successful axonal regeneration in vitro over neurocan, a well-known axonal growth-inhibitory CSPG. We now propose to study if an increased L1.1 level in brainstem neurons would enhance their ability to regenerate their axon over neurocan in vitro. We will employ our established brainstem neuron culture system and adeno-associated viral vectors to increase L1.1 levels. The degree of axon growth will be determined using quantitative (unbiased) assessment techniques. We predict that higher L1.1 levels further enhance axon growth from brainstem neurons over neurocan, especially from those that otherwise were unsuccessful. The results from our experiments will allow testing our

premise that *increasing L1.1 within brainstem neurons elicits axon regeneration over an inhibitory neurocan environment.*

Milestones:

Specific Aim 1:

During this review (Mo. 13-24) period, we have continued our development of a zebrafish neurocan secreting primary zebrafish CNS cell population. As mentioned in previous quarterly reports we have been able to obtain a putative Neurocan product secreted from a 293T cell line. Based upon consultation from experts in the field of glycobiology, we have re-focused our attention to develop the neurocan product produced in zebrafish cells in order to obtain a product with zebrafish-specific glycosylation.

During the past year, we continued to work towards subcloning Myc-NcanB into a pCS2+ plasmid vector. In parallel, we are also subcloning Myc-NcanB into a pAAV-2A-eGFP vector backbone. Our rationale for this cloning experiment follows. First, we have previously demonstrated that pAAV-2A-eGFP plasmids can successfully transfect and express proteins in our zebrafish brainstem cultures (Figure 1). Secondly, the eGFP reporter allows us to observe transfection efficiency. Previous experiments using our anti-c-Myc antibody for immunocytochemistry revealed that zebrafish brainstem cells endogenously express Myc. Hence, cellular Myc-NcanB protein expression cannot be accurately determined using anti-c-Myc. We currently do not have an antibody that specifically recognizes zebrafish NcanB.

During this review period, we completed cloning Myc-NcanB into the pAAV-2A-eGFP expression vector. Sequencing confirmed that the construct was in-frame. As a back-up plan we are also finalizing cloning Myc-NcanB into the pCS2+ vector backbone, and plan to transfect the new Myc-NcanB expression constructs into zebrafish cells

Now that we have cloned Myc-NcanB into the new expression vectors, we will examine protein expression of both constructs using Western blots. The construct with the highest protein expression will be used to produce purified Myc-NcanB. We will then determine an optimal concentration of Myc-NcanB for use as a substrate and finish quantifying the response of neurons to Myc-NcanB. This will generate a baseline for the response of untreated neurons to Myc-NcanB. We will then go on to analyze the effect of increased L1.1 levels in neurons exposed to a neurocan substrate.

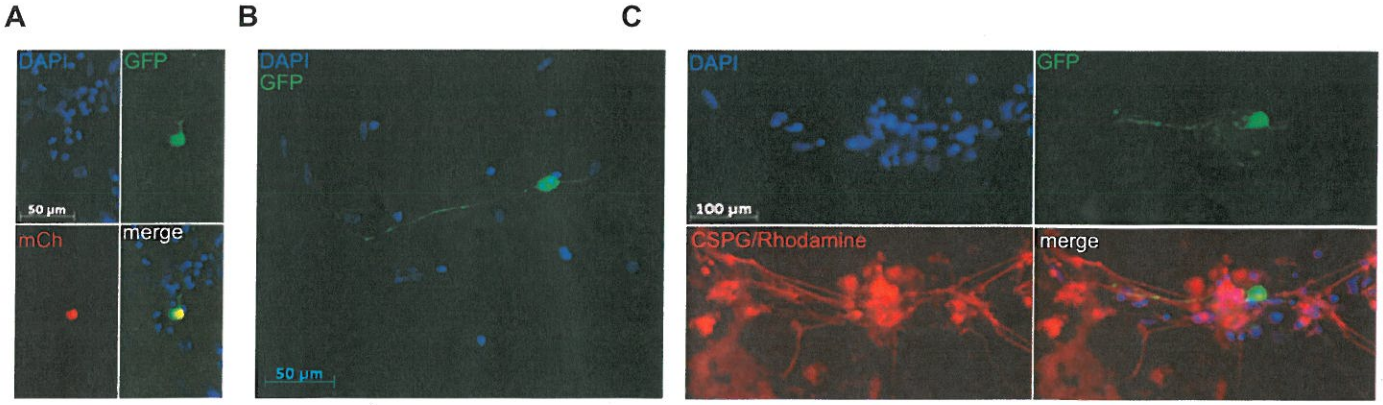

Figure 1. A. pAAV-mCherry-2A-eGFP transfected cell plated on laminin. Individual images from three fluorescent channels and merge. DAPI labels nuclei. GFP and mCherry genes are expressed off the transfected construct. B. pAAV-nadl1.1-2A-eGFP transfected cell plated on laminin. GFP demonstrates construct is expressed. C. pAAV-nadl1.1-2A-eGFP transfected cell interacting with CSPGs, labeled with rhodamine-dextran (red). Individual images from three fluorescent channels and merge.

SOW Plunkett Lab:

Specific Aim 2: To determine the involvement of PTPσ in regeneration from descending adult zebrafish brainstem neurons.

PTPσ is a recently discovered receptor of CSPGs. We showed that PTPσ mRNA is present in adult zebrafish CNS and studied its involvement in axon growth from cultured adult zebrafish brainstem neurons. We now propose to investigate the contribution of PTPσ to axon regeneration *in vivo*. We will use morpholino-techniques to reduce PTPσ levels in brainstem neurons that project their axon into the spinal cord and assess their regenerative capacity. The extent of axonal regeneration beyond areas of CSPGs within the injured spinal cord will be assessed. We predict that axon regeneration beyond a spinal cord injury is reversely correlated to the availability of PTPσ. Less PTPσ will increase the growth response of axons. The outcomes of these studies will allow testing our premise that *down regulation of PTPσ in adult zebrafish brainstem neurons promotes axonal regeneration in the injured spinal cord.*

Milestones:

Specific Aim 2.

***Experimental approach**: We will knockdown expression of PTPσ within transected descending brainstem neurons using PTPσ antisense morpholino. Zebrafish will receive a complete spinal cord transection and Gelfoam containing PTPσ antisense morpholino. In parallel, control groups will receive control morpholino or Gelfoam alone. Axonal regeneration in the spinal cord and the presence of neurons in a regenerative state will be assessed quantitatively. Together these outcome measures will allow evaluating the effect of PTPσ knock-down on axonal regeneration. Functional (swimming) recovery will be assessed through analysis of a number of parameters related to swimming behavior. Functional recovery will be correlated with axonal regeneration.*

Preparing for baseline experiments

During this past year review period, we continued in vivo morpholino experiments in adult zebrafish subjected to spinal cord injury. Gelfoam saturated with a control morpholino, nadl1.1 morpholino or a predicted translation blocking ptprs morpholino (5'-TGACGCAGATGACCTTTGACCTGGC-3') were introduced into the spinal cord of adult zebrafish at the time of spinal cord injury. We hypothesize that compared to control morpholino treated animals, nadl1.1 treated animals will have impaired locomotor recovery after spinal cord injury, while ptprs morpholino treated animals will have improved locomotor recovery after spinal cord injury. We have performed multiple experiments to date and the results thus far are inconclusive. We are currently confirming potential knockdown of PTPσ and below is described our strategy.

ptprs morpholino (ptprsMO) knock down confirmation

During this past year, we successfully cloned the ptprs morpholino (MO) binding site upstream and in-frame of GFP cDNA in pCS2+ vector backbone. The identity of our new ptprsMO-GFP pCS2+ construct was confirmed by DNA sequencing. We plan to begin testing the efficacy of the ptprs morpholino within the next month. The construct will be transfected into zebrafish cells treated with either the ptprs or control morpholino. GFP expression would indicate the morpholino does not bind its target sequence and cannot knockdown protein translation. This result would lead us to design and test a new morpholino to knock down ptprs protein expression. In contrast, GFP knock down would demonstrate the morpholino is capable of recognizing ptprs mRNA and blocking translation. Similar strategies have been used to validate morpholino target specificity (Bresciani et. al., 2010).

Functional (swimming) Recovery Analysis

During this 12 month review period, we began analyzing functional (swimming) recovery data in untreated and morpholino-treated spinal cord injured animals using our new video-tracking system. As shown in figure 2, we calculated an approximate average swimming distance of 2200 cm during a 5 minute period in uninjured fish. An approximate 4-fold reduction in swimming distance was observed through two weeks following SCI. Data for control morpholino-treated spinal cord injured animals has been collected and currently being analyzed and compaired to SCI alone fish. Once confirmation of knockdown PTPσ is complete (see above) we will examine the behavioral consequences of PTPσ morpholino delivery following SCI.

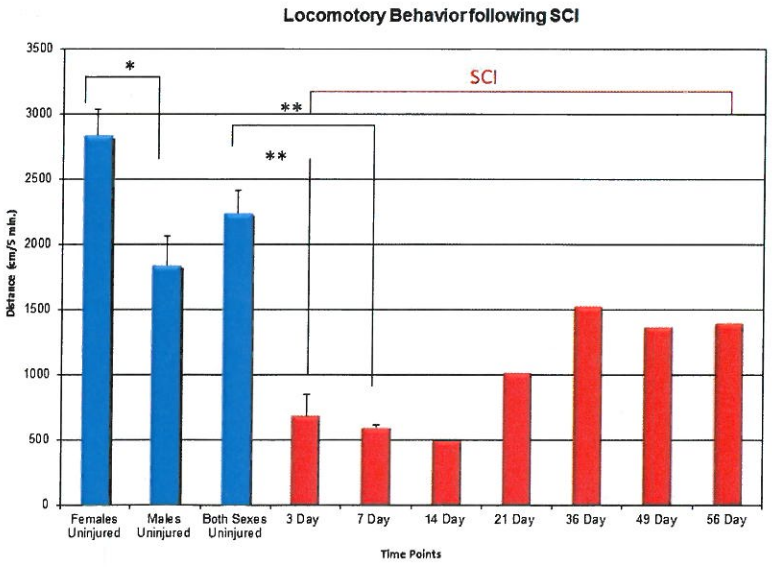

Figure 2. Unstimulated swimming distance by uninjured and spinal cord injured (SCI) adult zebrafish over a 5 minute interval. The number of days post-SCI are listed. Data obtained from uninjured male (*n=12*) and female (*n=18*); 3-day (*n=6*), 7-day (*n=3*) and 14-day SCI (*n=2*) fish. To date only a single fish was measured for groups: 21, 36, 49, and 56-day SCI. Error bars refer to the standard error of the mean (SEM). * $p<0.05$; ** $p<0.01$ based on a One-way ANOVA test, followed by a Turkey-Kramer Multiple Comparison post-test using InStat software.

SOW Oudega Lab:

Specific Aim 3: To determine the effects of over expression of genes involved in axonal extension in the transected adult zebrafish spinal cord on axonal regeneration and functional restoration.

Genetic deletion of identified genes involved in axonal regeneration impairs brainstem neurons to extent their axon beyond a CSPG-rich spinal cord transection site. We now propose to over express genes involved in axonal regeneration in adult zebrafish and investigate the effect of this genetic manipulation on axonal regeneration beyond a spinal cord transection. We will determine the degree of axon growth and accompanying functional recovery using tracing, functional testing, and histology. We predict that over expressing the genes will enhance the overall axonal growth process and will be accompanied by improved functional restoration. The results from these studies will allow testing our hypothesis that *axon regeneration from adult zebrafish brainstem neurons depends on the expression of a specific set of genes.*

Milestones:

Specific Aim 3.

Trace brainstem neurons/regenerating axons. Analyze functional data.

Fluoro-emerald and fluoro-ruby are used to trace brainstem neurons in adult zebrafish with transected spinal cord. For proper gene analysis we need to trace 1500 fish to obtain about 50000 brainstem neurons per population (i.e., regenerating and non-regenerating neurons). We are on our way to collect these back-labeled neurons and perform our analysis. In the meantime, we tested RNA quality after various fixation protocols including zinc-fixation. We have optimized the protocol to yield the highest quality of RNA possible to reduce the possibility of false positive or negative data. A manuscript on this optimization process is in preparation. Will awaiting the survival of the fish and the collection of the traced neurons, which will take time due to the required high number of fish, we have tested our procedures to isolate high quality RNA. A number of different protocols were tested as these have all different effects on neurons from different species. Mostly rat-protocols were used as there are no standard protocols for zebrafish at present. Some variations of an acquired zebrafish protocol were also tested. We have now identified a protocol that yields high quality RNA from zebrafish neurons. A report on the time-course of retrograde labeling of regenerated brainstem neurons and functional (swimming) ability is being prepared for submission to a peer-reviewed scientific journal. Tracer was injected at different times (2-8 weeks) after a complete transection of the spinal cord. Swimming ability was assessed. Tissue was processed and labeled neurons quantified to reveal: (1) the optimal time for tracer injection for our RNA-collection and microarray study; (2) the relationship between regenerating axons and return of swimming ability; (3) the type of neurons that area able to regenerate across the injury into the spinal cord.

Key Research Accomplishments Specific Aims 1 and 2, Plunkett Lab:
Specific Aim 1:

1. Completed cloning Myc-NcanB into the pAAV-2A-eGFP expression vector.

2. Completed cloning Myc-NcanB into the pCS2+ vector.

3. Transfection of the new Myc-NcanB expression constructs into zebrafish cells is underway.

Specific Aim 2

1. Delivery methods for morpholino delivery were established.

2. Retrograde neuronal tracing techniques were established in the Plunkett lab to label brainstem neurons transected by spinal cord injury.

3. PTPσ morpholino knockdown confirmation construct was developed. This construct contains the ptprs morpholino binding sequence upstream of GFP (ptprsMO-GFP pCS2+).

4. Have established behavioral methodologies through functional swimming tests.

Key Research Accomplishments in Specific Aim 3, Oudega Lab :
Specific Aim 3

1. Completed harvesting regenerating and non-regenerating neurons from brainstem of injured zebrafish.

2. Isolated and determined quality of RNA from both types of neurons.

3. Determined upregulated GAP-43 RNA in regenerating neurons to assess their regenerative state.

4. Microarray are being processed.

Reportable Outcomes:

Publications:

1. Katerina Vajn, Jeffery A Plunkett, Alexis Tapanes-Castillo and Martin Oudega.
Axonal regeneration after spinal cord injury in zebrafish and mammals: differences, similarities, translation. Neurosci Bull 2013; 29(4):402-10.

2. Alexis Tapanes-Castillo, Francelethia Shabazz, Mam M'boge, Katarina Vajn, Martin Oudega, and Jeffery A. Plunkett.
Characterization of a novel primary culture system of adult zebrafish brainstem cells.

Conditionally accepted Journal of Neuroscience Methods.

3. Katarina Vajn, Denis Suler, Alexis Tapanes-Castillo, Jeffery A Plunkett, Martin Oudega.
Temporal profile of endogenous spinal cord repair in the adult zebrafish.

Manuscript in preparation.

Posters, presentations and Meetings:

Three undergraduate students from Miami-Dade Honors College conducted research in the Plunkett lab from June-August 2012 through the SRI. They submitted 2 posters and gave one oral presentation at the ***1st Annual Miami Dade College STEM Research Symposium*** *in September 2012. Jonathon Lehrman a Plunkett lab student won Best Oral Presentation.*

Four undergraduate students presented two Plunkett lab posters at the ***2012 University of Miami, Miller School of Medicine Neuroscience Research Day Symposium****.*

-Three posters were presented at the ***Society for Neuroscience Annual Meeting*** *in New Orleans, LA in November 2012. Five undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.*

- ***January 2013, 46th Annual Winter Conference on Brain Research.*** *A panel session organized by* ***Dr. Jeff Plunkett*** *featured talks from* ***Dr. Martin Oudega*** *(Univ of Pitt),* ***Dr. Herb Geller (NIH), Dr. James Fawcett (Univ. of Cambridge)*** *and* ***Dr. Plunkett*** *on CSPGs. Three posters from the Plunkett/Oudega labs, were also presented. Six undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.*
Winter Brain Panel
CSPGs in CNS injury and repair: a mammalian and non-mammalian perspective.
James W. Fawcett, Herbert M. Geller, Martin Oudega, Jeffery A. Plunkett

Winter Brain Posters

Analysis of putative stem and neural progenitor cell populations following CNS injury in the adult zebrafish

Jeffery Plunkett, Francelethia Shabazz, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega

Characterization of a novel primary neuronal culture from adult zebrafish brainstem.

Alexis Tapanes-Castillo, Francelethia Shabazz, Isaac Chacon, Jossias Genao, Arjena Valls, Katarina Vajn, Martin Oudega, Jeffery Plunkett

Keystone Conference "Growing to Extremes: Cell Biology and Pathology of Axons", Tahoe City, CA in March 10-15, 2013.

A. Tapanes-Castillo, F. Shabazz, I. Chacon, J. Genao, A. Valls, K. Vajn, M. Oudega, J.A. Plunkett.

Primary neuronal cultures from the brainstem of adult zebrafish: a novel *in vitro* tool to study axonal growth across inhibitory chondroitin sulfate proteoglycans.

Comment: Dr. Tapanes-Castillo was awarded a $1,200.00 NIH travel fellowship to attend this meeting. Three undergraduate students from the Plunkett lab were granted authorship due to their significant contributions to the work.

Barry University STEM Science Symposium, Miami, FL. March 27th, 2013

Isaac Chacon, Francelethia Shabazz, Jossias Genao, Arjena Valls, Katarina Vajn, Martin Oudega, Alexis Tapanes-Castillo, Jeffery Plunkett.

Characterization of a novel primary neuronal culture from adult zebrafish brainstem.

Comment: Isaac Chacon an undergraduate student in the Plunkett Lab won second place overall for poster presentation.

The following four posters were presented at 8th Annual Southeast Cell Science Undergraduate Research Symposium. Miami, FL April 20, 2013.

Ibis Iser, Haydee Torres, Francelethia Shabazz, Alexis Tapanes-Castillo, Katarina Vajn, Martin Oudega and Jeffery A. Plunkett.

The effect of reducing protein tyrosine phosphatase receptor (ptprs) protein levels on functional recovery after spinal cord injury

Arjena Valls, Alexis Tapanes-Castillo, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Jeffery Plunkett.

The Effects of Zebrafish Neurocan and its Putative Receptor, Protein Tyrosine Phosphatase, on Neurite Outgrowth *in vitro*

Alcides Lorenzo Gonzalez, Aileen Hernandez, Alejandra Cartagena, Lisandra Yut, Francelethia Shabazz, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega, Jeffery Plunkett.
The role of central nervous system stem cells in adult zebrafish brainstem neuron axon regeneration
Comment: Won best poster presentation

Isaac Chacon, Francelethia Shabazz, Katarina Vajn, Martin Oudega, Alexis Tapanes-Castillo, Jeffery Plunkett
Behavioral response of adult zebrafish brainstem cells to inhibitory Chondroitin Sulfate Proteoglycans (CSPGs) *in vitro*

Neuroscience 2013
One poster will be presented at the Society for Neuroscience meeting in San Diego CA

Jeffery Plunkett, Francelethia Shabazz, Isaac Chacon, Katarina Vajn, Alexis Tapanes-Castillo, Martin Oudega
The effect of a pre-conditioning spinal cord lesion on the response of adult zebrafish brainstem cells to inhibitory chondroitin sulfate proteoglycans (CSPGs) *in vitro*.

Other outcomes:

- *Undergraduates Harold Gomez (Michigan) and Anthony Wood (Morehouse) from the **Plunkett Lab** were accepted into medical school for the Fall 2013 semester. Undergraduate Emer Bajuelos from the Plunkett lab was accepted into NYU dental school for the Fall 2013 semester. Undergraduates Jossias Genao, Alejandra Cartagena, and Lisandra Yut graduated December 2012 with bachelor degrees in Biology and all plan to attend medical, dental or graduate school.*

Conclusion:
The different studies within this proposal (*in vitro* as well as *in vivo*) have been progressing reasonably well according to the described milestones. Some technical/experimental barriers were encountered and these needed to be overcome. This was accomplished for most of them and is still in progress for few. Considering our previous success with surmounting these roadblocks, we are confident that we will be successful. Thus, in conclusion, we are well on our way to accomplish the goals for this year as they were described in our proposal

References:
Bresciani E, Confalonieri S, Cermenati S, Cimbro S, Foglia E, Beltrame M, DiFiore PP, Cotelli F. (2010) Zebrafish numb and numblike are involved in primitive erythrocyte differentiation. PLoS One Dec 13;5(12) 1-10.

CUMULATIVE EXPENDITURE REPORT
ST. THOMAS UNIVERSITY – Sept. 30, 2013

1. Award No. ____W81XWH-11-1-0645_____2. Report Date _9/30/2012 (annual)

3. Reporting period from __6/1/12_____ to ___8/31//13_____

4. PI ___Dr. Jeffrey Plunkett_____ 5. Telephone No. (305) 628-6572_____

6. Institution_____St. Thomas University_____

7. Project Title____Molecular Determinants Fundamental to Axon Regeneration after SCI

8. Current staff, with percent effort of each on project.

Dr. Jeffrey Plunkett, PI	25% (academic year) + 100% (summer)
Dr. Martin Oudega, Co-PI	40%
Dr. Alexis Tapanes-Castillo (post doc Plunkett lab)	100%
Fran Shabazz (research technician, Plunkett lab)	100%
Katarina Vajn (Post Doc Oudega lab)	100%

9. Award expenditures to date (as applicable):

	This Qtr/Cumulative		This Qtr/Cumulative
Personnel	$43,231.41/ $ 271,073.45	Travel	$0/ $28,477.41
Fringe Benefits	$11,045.02 / $73,549.49	Equipment	$1,899.00 / $10,111.45
Supplies	$7,535.07 / $39,074.56	Other	$2,542.18/$16,257.68

	This Qtr/Cumulative
Subtotal	$66,252.68 / $438,544.04
Indirect Costs	$27,809.87/ $186,111.15
Fee	875.00 / 1,784.00
Total	$94,937.55 / $626,439.19